PROTEIN THAT MODULATES THE STABILITY OF TRANSCRIPTIONAL
REGULATORY COMPLEXES REGULATING NUCLEAR HORMONE RECEPTOR
ACTIVITY, DNA ENCODING SAME, AND ANTIBODIES THERETO

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CROSS-REFERENCE TO RELATED APPLICATION

[0002] The present application claims priority under 35

U.S.C. §119(e) from U.S. provisional application no.

15   60/248,191, filed November 15, 2000, the entire contents of

which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0003] The present invention relates to molecules that

20   interact with proteins regulating the nuclear hormone receptor

family of transcription factors.

Description of the Related Art

[0004] All of the cells of the lymphohematopoietic system

can be generated from a single cell (Nowell et al, 1969;

Spangrude et al, 1988; Capel et al, 1990; Jordan et al, 1990; Wu et al, 1968). This pluripotential hematopoietic stem cell (PHSC) is separated from the mature cells of the peripheral blood by a series of intermediate cells of increasingly

5 restricted developmental potential (Hodgson et al, 1979; Magli et al, 1982; Eaves et al, 1992; Jones et al, 1990; Lansdorp et al, 1990). The most restricted are committed to a single lineage and have limited capacity for self-renewal. Recombinant growth factors and clonal culture systems have

10 made it possible to identify and isolate some committed progenitors, but the more immature PHSC has proven more elusive. Neither the exact number of PHSC nor the process by which their number is maintained is known and human PHSCs have not been unambiguously identified (Orlic et al, 1994). Most

15 human hematopoietic progenitors express the CD34 antigen on their surface and this marker has been used extensively to identify and isolate hematopoietic progenitors (Krause et al, 1996). A CD34+CD38- fraction, which constitutes 0.1% of freshly isolated bone marrow cells, contains most of the PHSC

20 activity in normal marrow (Terstappen et al, 1991). PHSC are not undifferentiated cells, but are uniquely specialized cells, whose role is to provide progenitors for the various hematopoietic lineages in a demand-responsive manner, while protecting the stem cell pool from depletion. The molecular

basis for this ability is not known but is under intense
scrutiny.

[0005] Several approaches have been used to try to identify
the genes whose products regulate the stem cell pool. Yang et
al (1996) made an EST database for CD34+ cells by single pass
sequencing of 402 clones from a directional library. Thirty-
five percent of the sequences were from previously unknown
genes but none of these were differentially expressed in PHSC.
Graf et al (1995), using differential display to identify
differences between $CD38^{HI}$ and $CD38^{LO}$ cells, identified one
previously unidentified sequence ("345"), that was expressed
at ~2.5 times higher concentration in the $CD38^{LO}$ population.
The sequence contained no open reading frame and lacked a
polyadenylation site. The pace of the search for
hematopoietically relevant genes has quickened lately. Using
a cDNA library prepared from CD34+ cord blood cells, close to
10,000 ESTs were identified (Mao et al, 1998). The majority
of these were either known sequences (47.6%) or corresponded
to previously catalogued ESTs (26.4%), but 14.3% were new
ESTs. A retroviral gene trap vector that selects for
integration in or near expressed 5' exons has also been used
in an attempt to identify genes that were repressed during
hematopoietic differentiation (Muth et al, 1998). Two genes,

of unknown function, were identified but targeted deletions failed to show hematopoietic abnormalities.

[0006] In all living creatures, cells are continuously dying. They are either killed by injurious agents or they are induced to commit suicide. Cells that are damaged by injury, such as by mechanical damage, exposure to toxic chemicals, undergo a characteristic series of changes: they swell (because the ability of the plasma membrane to control the passage of ions and water is disrupted) and the cell contents leak out, eliciting an inflammatory response in the surrounding tissues. Cells that are induced to commit suicide shrink, have their mitochondria break down with the release of cytochrome c, develop bubble-like blebs on their surface and undergo DNA and chromatin fragmentation. Ultimately they break into small, membrane-wrapped, fragments which are engulfed by nearby phagocytic cells without causing inflammation. The pattern of events in death by suicide is called programmed cell death or apoptosis.

[0007] Programmed cell death is needed for remodeling of structures during development. Well-known examples include the resorption of the tadpole tail at the time of its metamorphosis into a frog; the formation of the fingers and toes of the fetus by removing (by apoptosis) the tissue

between them, and the sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation.

[0008] Programmed cell death is also required to destroy cells that represent a threat to the integrity of the organism. In the immune system, some cell-mediated killing of virus-infected cells occurs by inducing apoptosis and, as cell-mediated immune responses wane, the effector cells are removed by an apoptotic mechanism. Defects in the apoptotic machinery are associated with autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis.

[0009] Genetic damage can cause somatic cells to become malignant and lead to abnormal embryonic development (leading to birth defects). Cells respond to DNA damage by increasing their production of p53 which can induce apoptosis. Mutations in the p53 gene, producing a defective protein which does not induce apoptosis, are often found in cancer cells.

[0010] The decision to commit suicide depends on the balance between the positive signals needed for survival and signals initiating a death pathway (negative signals). The survival of many cells requires that they receive continuous stimulation from other cells and, for many, continued adhesion to the surface on which they are growing. In the absence of these positive signals, cells initiate a program leading to cell death. Cellular damage by increased levels of oxidants,

damage to DNA by these oxidants or other agents like ultraviolet light, X-rays and some chemotherapeutic drugs, as well as agents that bind to specific receptors on the cell surface, also initiate the apoptosis program. These death activators include tumor necrosis factor (TNF)and lymphotoxin that both bind the TNF receptor; and Fas ligand (FasL), a molecule that binds to Fas (CD95).

[0011] Cells appear to use different pathways to initiate apoptosis depending on the source of the signal. In a healthy cell, the outer membranes of mitochondria, the endoplasmic reticulum (ER) and the nuclear envelope express the protein Bcl-2 on their surface. Bcl-2 binds a molecule of Apaf-1, which is itself bound to a molecule of caspase 9. Caspase 9 is one of a family of over a dozen caspases (cysteine-aspartate proteases). Internal damage in the cell results in release of cytochrome c from the mitochondria and causes Bcl-2 to release the heterodimer of Apaf-1 and caspase 9. These aggregate in the cytosol. Caspase 9 cleaves and, in so doing, activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity that leads to digestion of structural proteins in the cytoplasm degradation of chromosomal DNA and death of the cell.

[0012] Apoptosis can also be triggered by external signals. Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary death activator (FasL and TNF, respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to cell death.

[0013] Several known proteins or families of proteins that function to regulate apoptosis include:

1. Members of the BCL-2 family. Some members of this family inhibit apoptosis, including Bcl-2 and Bcl-x. Others, such as BAX, stimulate apoptosis. BAX is thought to accomplish this by binding to and inhibiting the anti-apoptotic functions of Bcl-2 and Bcl-x.

2. FLAMES 1 and 2 are proteins that regulate cell death mediated by receptors of the TNF receptor family. TRAIL receptors are cell death receptors which are members of the TNF receptor family and exert cell suicide effects on cancerous but not normal cells.

3. IAP family members are homologous to the baculovirus IAPs. The open reading frames (ORFs) possess three baculoviral inhibition of apoptosis protein repeat (BIR) domains and a carboxy-terminal RING zinc-finger. The human

IAP genes have a distinct but overlapping pattern of expression in fetal and adult tissues. These proteins significantly increase the number of known apoptotic suppressors. A fourth member of the family, termed *survivin* has also been identified. All appear to be caspase inhibitors.

[0014] IEX-IL is an nf-kB regulated survival factor, that protects cells from Fas or TNF induced apoptosis.

[0015] The nuclear hormone receptor (NHR) superfamily is a large family of mainly ligand-dependent transcription factors that play a role in the regulation of reproduction, growth, differentiation, and homeostasis. The family includes receptors for steroid hormones (estrogen, androgen, adrenal glucocorticoid, aldosterone, and progesterone), thyroid hormone and retinoic acid as well as a group of so-called "orphan receptors" that have poorly, defined ligand(s).

[0016] Members of the family share several structural features including a conserved DNA binding domain (DBD) that targets the receptor to specific DNA sequences which are called hormone response elements (HRE). The carboxyl-end of the receptor contains the ligand-binding domain (LBD) and embedded within this LBD is a hormone-dependent transcriptional activation domain. The LBD serves as a molecular switch that recruits co-activator or co-repressor proteins that regulate transcription of the target genes.

Ligand-dependent receptors like the thyroid hormone receptor (T3R) and retinoic acid receptor (RAR) stimulate transcription when ligand is bound and repress it when the ligand is absent (Hu et al, 2000).

[0017] The best-characterized mammalian co-repressors are N-CoR (nuclear receptor co-repressor) (Chen et al, 1995) and SMRT (silencing mediator of retinoid and thyroid receptor) (Horlein et al, 1995). These co-repressors fill overlapping but non-redundant roles in regulating transcription. Both SMRT and N-CoR are large proteins (>170 kD) that exist in multi-protein complexes that have an estimated size of 1.5-2 mDa. A SMRT complex, isolated by a combination of conventional and immunoaffinity chromatography has been shown to contain histone deacetylase 3 (HDAC3) and transducin (beta)-like I (TBL1), a WD-40 repeat-containing protein (see below). The HDAC3-containing, SMRT and N-CoR complexes can bind to unliganded thyroid hormone receptors (T3Rs) *in vitro* (Li et al, 2000). Although both co-repressors are expressed widely, extensive hematological abnormalities including blocks in erythrocyte and T-cell development (Jepsen et al, 2000) follow targeted deletion of N-CoR.

[0018] Co-repressors mediate transcriptional silencing by inhibiting the basal transcription machinery or by recruiting chromatin-modifying enzymes (Hu et al, 2000; Li et al, 2000; Wong et al, 1998; Burke et al, 2000). Histone deacetylation, which

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produces a more compact chromatin structure that is inaccessible to transcriptional activators (Burke et al, 2000), appears to be the predominant means of chromatin modification. Studies of RAR and T3R show that ligand binding leads to the displacement of an HDAC-

5   containing complex from the nuclear receptor in exchange for a histone acetyltransferase (HAT)-containing complex and this may serve as a general mechanism for switching nuclear receptors from a transcriptionally repressive to a transcriptionally active state (Xu et al, 1999). Transcriptional repression by N-CoR involves a

10   co-repressor complex that contains one or more HDAC and may include mSin3A/B (Huang et al, 2000). Changes in repression correlate with alterations in the level of N-CoR and/or SMRT. These levels are regulated by both the rate of synthesis of the co-repressors and, more dramatically, by their rate of degradation. Targeted

15   proteolysis of transcriptional co-regulators has been established as a mechanism for cell-specific regulation of gene transcription (Zhang et al, 1998a). Although the composition of the repressor complex is not fully understood a protein called TBL1 is present in some cells (Huang et al, 2000; Guenther et al, 1998) and in these

20   cells, the extent of transcriptional repression was regulated by the amount of TBL1 present.

[0019] Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the

present application. Any statement as to content or a date of

any document is based on the information available to

applicant at the time of filing and does not constitute an

admission as to the correctness of such a statement.

5   SUMMARY OF THE INVENTION

[0020] The present inventor has discovered a gene in humans

and in mice, designated C21, which encodes a family of

proteins that play a role in transcriptional regulation. Two

isoforms (α and β) produced by alternative splicing have been

10   identified in humans. A transgenic model was created that shows

that over-expression of C21 in mouse hematopoietic cells alters

myeloid development and suggests that members of this family are

involved in regulating stem cell differentiation. Over-expressing

C21 in 3T3 fibroblasts increases their resistance to apoptotic

15   stimuli. The C21 protein forms a complex with a class of molecules

that plays a critical role in transcription, the co-repressors of

the nuclear hormone receptors (NHR). These co-repressors mediate

the down-regulation of gene expression. C21 binds to the co-

repressors and appears to interfere with the ubiquitin-mediated

20   proteolysis of the co-repressors, causing an elevation of the co-

repressor concentration. Members of the family are expressed at

high levels in fetal hematopoietic tissues as well as in many

hematopoietic cell lines. Like many WD40 proteins, C21 family

members appear to act by serving as an adaptor or bridge,

facilitating the interaction of proteins that do not interact
directly.

[0021] The present invention thus provides a C21 polypeptide,
which interacts with, and has the activity of modulating the
stability of, transcriptional regulatory complexes that regulate
nuclear hormone receptor activity, and fragments of C21 polypeptide
that retain this activity. Also comprehended by the polypeptide
according to the present invention is a variant of the C21
polypeptide that has an amino acid sequence with at least 85%
sequence identity to the C21 polypeptide of SEQ ID NO:2, and which
interacts with, and also has the activity of modulating the
stability of, transcriptional regulatory complexes that regulate
nuclear hormone receptor activity.

[0022] The present invention further provides a molecule
having the antigen-binding portion of an antibody specific for
the polypeptide according to the present invention.

[0023] Other aspects of the present invention are directed
to a nucleic acid molecule encoding the polypeptide, fragment
or variant thereof, of the present invention, a vector
containing this nucleic acid molecule, and a host cell
transformed with the nucleic acid molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figures 1A-1D are dot plots (two-dimensional
histograms) illustrating the purification of CD34/+CD38- and

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CD34+/CD38+ cells from human bone marrow (BM). The abscissa represents the fluorescence intensity of the staining with anti-CD38 coupled to fluorescein and the ordinate the intensity of the staining with anti-CD34 coupled to

5   phycoerythrin. Figure 1A is the starting BMC (3.2% CD34+). Figure 1B is after removal of lineage positive cells (72% CD34+). Figure 1C is sorted CD34+/CD38- cells (99% CD34+, 97% CD38-). Figure 1D is sorted CD34+/CD38+ cells (96% CD34+, 98% CD38+).

10   [0025] Figures 2A (virtual Northern blots) and 2B (RT-PCR analysis) show differential expression of C12, C21, C23 and C40 by CD34+/CD38- and CD34+/CD38+ BMC. In the blots of Figure 2A, double-stranded cDNAs amplified from total RNA obtained from the CD34+/ CD38- and CD34+/CD38+ cells were used

15   to demonstrate expression rather than conventional Northern blots because of the paucity of RNA available. The cDNAs were electrophoresed through a 1% agarose gel, transferred to nitrocellulose membranes and hybridized with C20, C12, C21, C23, C40 and β-actin probes respectively. The resultant

20   autoradiographs were scanned and their digitized images are shown in the figure.

[0026] In the RT-PCR analysis of Figure 2B, ethidium bromide stained agarose gel electrophoresis of reaction products are shown. Preparations from CD34+/CD38- and

CD34+/CD38+ cells were placed in adjacent lanes and are
labeled CD38- and CD38+. The RT-PCR was performed with total
RNA extracted from ~35000 sorted, lineage negative CD34+/CD38-
and CD34+/CD38+ BMC. Each sample was amplified with primers
5   for C12, C21, C23 and C40 as well as primers that would
amplify the cDNAs of CD38 and actin, which were included as
controls. Each pair of lanes is labeled to indicate the
primer pairs used in the amplification. Although the same
number of CD38- and CD38+ cells were used for the RNA
10 preparations, analysis of the photographic image indicates
that there was ~1.8 times as much actin message in the
CD34+/CD38+ sample than in the CD34+/CD38- sample. Despite
this, all four cDNAs are more abundant in the reaction
mixtures prepared from the CD34+/CD38- cells. CD38, as
15 expected was more abundant in the CD34+/CD38+ cell population.
The PCR was performed with specific primers selected to
amplify fragments that could be distinguished from each other
on the basis of the size of expected product (C12 (500 bp),
C21(156 bp), C23 (151 bp) and C40 (192 bp)). Primers for actin
20 (668 bp) and CD38 (236 bp) were synthesized from published
sequences. The amplification was performed under the following
conditions: 1 minute at 94°C followed by 28 cycles of 30
seconds at 94°C, 30 seconds at 58°C and 2 minutes at 72°C with
a final 10-minute extension at 72°C.

[0027] Figures 3A-3D show the expression of mRNA encoding
C12, C21 and C23 by human tissues. Multiple tissues dot
blots, purchased from Clontech, were hybridized with ^{32}P-
labeled probes. In each case the probe used was the original
fragment cloned after the PCR-mediated subtractive
hybridization. The same filter was used for all
hybridizations. C23 was hybridized first (Figure 3B), then
C21 (Figure 3C) and finally C12 (Figure 3D). Between
hybridizations the bound radioactive probes were stripped and
the filter exposed to fresh film to assure that the stripping
had been complete. The template shown in Figure 3A identifies
the source of the RNA in each position. The autoradiographs
were scanned to produce the images shown. The signals
obtained with the three neural tissues RNA were cut from the
original image and moved to the row D, positions 6, 7 and 8.

[0028] Figures 4A-4C show the expression of C23, C21 and
C12 mRNA by hematopoietic cells. Figure 4A shows the
expression of C23 mRNA by peripheral white cells. Digital
reproduction of an autoradiograph showing the results of an
RNAse protection assay (RPA) to detect the expression of a
message that included the far 3' end of the 3' UTR.
Lymphocytes, monocytes and polymorphonuclear leukocytes were
isolated by flow cytometry from human peripheral blood based
on their light scatter properties. Total RNA from human brain

was used as positive control for C23. 18s rRNA was used as an internal control to normalize the amount of RNA added to each lane of the gel. In Figures 4B and 4C, the expression of C21 and C12 mRNA by human leukemic cell lines is shown. Digital reproduction of autoradiographs show the results of RT-PCR amplification of the ORF (1548 bp) of C21 (Figure 4B) and a 500 bp fragment of C12 (Figure 4C) in RNA extracted from human leukemic cell lines. In each case a single band of the appropriate molecular mass was detected.

[0029] Figures 5A-5B show the sequence of the cDNA encoding C21. The open reading frame is indicated in CAPITOL letters. The WD-sequence regions are shaded, and the potential phosphorylation site sequence is underlined.

[0030] Figure 6 is a schematic illustration of the strategy for cloning C21 cDNA. A human placental cDNA library was screened by PCR with primers from the original C21 sequence shown in the shaded box. The isolated clone (2180 bp) contained the entire 3' UTR shown in the solid black line. Sequential 5' RACE, using K562 double stranded cDNA as a template, revealed the 1545 bp ORF, indicated by the shaded line in the figure preceded by the 161 bp 5' UTR, indicated by the unshaded line.

[0031] Figure 7 shows the *in vitro* translation of the ORF of C21 mRNA. The ORF was amplified using a 5' primer that

began with the ATG start codon and a modified 3' primer that included a c-*myc* tag. The PCR product was ligated into PCR3.1 under the control of the T7 promoter. The vector was linearized with EcoRI, and mRNA was synthesized using T7 RNA

5 polymerase. This mRNA was used to support *in vitro* translation by a rabbit reticulocyte system which incorporated biotin labeled-lysine. The product was detected with streptavidin-horse radish peroxidase (HRP) and a chemiluminescent detection system (ECL). Both sense and

10 antisense messages were used to prime the translation system.

[0032] Figures 8A-8D show Western blotting with anti-peptide antibodies made against deduced sequences from C21 α and β. After transfer, the membranes were stained with polyclonal rabbit anti-C21 peptide antibodies and the

15 transferred proteins visualized with HRP-labeled donkey anti-rabbit IgG. The bound HRP was detected using luminol as the substrate.

[0033] Figures 9A-9H show that C21 is expressed by hematopoietic progenitors in fetal liver and adult marrow.

20 Figure 9A is C21α vs 17 Week Fetal Liver. Figure 9B is C21β vs 17 Week Fetal Liver. Figure 9C is C21α vs Adult Bone Marrow. Figure 9D is C21β vs Adult Bone Marrow. Figures 9E-9H are the same tissues (1-2 sections above or below) stained with pre-immune rabbit serum. Original Magnification is 40X.

Stained with HRP coupled goat anti-rabbit IgG. AEC chromagen.

Counter stained with Haematoxylin.

[0034] Figure 10 is an agarose gel showing C21 mRNA

expression by hematopoietic tumor cell lines. RT-PCR was used

to examine the expression of C21. The primers used amplified

only the α isoform and produced a 1546 bp fragment.

[0035] Figure 11 shows Northern blots of human tissue RNA

with either the 3' UTR or ORF of C21α as a ^{32}P-labeled probe.

[0036] Figures 12A and 12B show the expression of hC21 in

mouse fibroblasts in ribonuclease proteation assay (RPA;

Figure 12A) and Western blot (Figure 12B). The cDNA encoding

the ORF of hC21α was amplified by PCR, and the product ligated

in the sense configuration into pCDNA3.1. After

transformation of the appropriate *E. coli*, the plasmids were

isolated and used to transfect NIH3T3 cells using

lipofectamine. G418 resistant clones were selected and

analyzed for their expression of C21.

[0037] Figures 13A and 13B show that the expression of C21

increases the resistance of 3T3 fibroblasts to apoptosis

induced by serum starvation. The figure shows 2-dimensional

histograms (cytographs) of the staining of control 3T3-neo

(Figure 13A) and transfected 3T3-C21α (Figure 13B) cells with

propidium iodide (PI) and fluorescein-coupled Annexin V.

[0038] Figure 14 shows the intracellular localization of C21. Apoptosis was induced by either serum starvation (incubation with DMEM containing 0.2% FBS for 16 hours) or growth with 100 ng/ml of the topoisomerase inhibitor, camptothecin (CPT). The cells were washed, fixed with 3.8% paraformaldehyde, permeabilized with 0.0.5% Triton-PBS and blocked with 10% human serum in PBS. They were then incubated with the anti-C21 antibodies (1:25) and stained with FITC-F(ab')2 anti-rabbit IgG antibody that had previously been absorbed to remove reactivity with human immunoglobulins.

[0039] Figures 15A and 15B show GST-pull-down of *in vitro* synthesized C21 by SMRT (1-900). Hu SMRT(1-900) cDNA was amplified by PCR and cloned in-frame into pGEX-6p-1 (GST fusion expression vector for bacterial expression). Expression of the GST fusion protein (Figure 15A) or control (Figure 15B) was induced with IPTG and the product adsorbed onto glutathione sepharose 4B. hC21 (full-length ORF or fragments lacking the C-terminal half(δ C), the F-box (δ F), the N terminal 40 amino acid (δ N) or a construct lacking both the N-terminus and the F-box (δN+F) was cloned in-frame into pCDNA3 vector with a Flag tag at the N-terminus. The plasmid, linearized with EcoRI, was purified and used as a template to label the product. For the pull-down experiments, 5 ng of ^{35}S-labeled hC21 protein(s) and 20 ul of GST agarose or GST-SMRT agarose were mixed. All four C21 constructs

labeled with equal efficiency and the same quantity of labeled

protein were used in each experiment. The denatured proteins were

then electrophoresed through a 10% SDS PAGE gel.

[0040] Figure 16 shows that C21 co-precipitates with SMRT.

COS7 cells were transfected with pCDNA His-HDAC3 and either

pCDNA Flag-HC21 or an empty Flag vector. After 48 hours, the

cells were lysed in RIPA buffer. The lysates were cleared and

passed over a Ni-agarose column to adsorb the His-tagged HDAC3

and any associated proteins. After extensive washing, the

bound proteins were eluted with SDS-PAGE loading buffer and

analyzed. After transfer to nitrocellulose the SDS-PAGE-

separated proteins were stained with anti-Flag and anti-His

monoclonal antibodies.

[0041] Figure 17 shows that C21 expression increases the

expression of nuclear hormone receptor co-repressors. 36

hours after transfection the cells were lysed in SDS loading

buffer. Equal amounts of proteins were electrophoresed in

SDS-PAGE gel and blotted. Anti-His mAb was used to detect the

transiently expressed proteins. The C21 constructs used in this

experiment were also His-tagged and are visible on the blot after

staining. They are marked with * in the figure. The

immunoprecipitates were quantified by phosphorimaging. After

stripping, the blots were re-stained with anti-ERK as a

control for loading differences.

[0042] Figure 18 shows that C21 stabilizes SMRT. 293T cells were transfected with PcDNA His-SMRT(1-300) and either C21 or an empty vector using CaPO4 mediated transfection. After 36 hours, the cells were pulse labeled for 1 hour with 0.1 mCi of

5 [^{35}S]methionine, and the label chased with cold media. At the end of the labeling period and again after 1 and 3 hours, the cells were sampled, lysed in RIPA buffer, and the His-tagged proteins isolated with Ni-agarose. The eluted proteins were subjected to SDS-PAGE, and the dried gels were used to expose X-ray film.

10 [0043] Figure 19 shows the effect of proteasome inhibition on SMRT expression. COS7 cell, expressing SMRT(1-900) were treated with 15 um MG132 for 10 hours. The His-tagged SMRT was detected by Western blotting.

DETAILED DESCRIPTION OF THE INVENTION

15 [0044] The present invention is based on the discovery of a single C21 gene which encodes a family of proteins that can be detected by Western blotting and that plays a role in transcriptional regulation. The heterogeneity is due to the presence of multiple isoforms, produced by differential exon

20 utilization. Although the ORF of the predominant form contains only 1545 bp, the gene occupies ~100 kb of genomic DNA on chromosome 3q and contains 16 exons. C21 has significant homology (79%) to only one other vertebrate gene, TBL1. Both C21 and TBL1 interact with proteins that regulate the nuclear hormone receptor

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family of transcription factors. C21α co-precipitates with a co-
repressor of nuclear hormone receptors (NHR), SMRT (silencing
mediator of retinoic acid and thyroid hormone receptors), in pull
down experiments, and co-precipitates in complexes

5   immunoprecipitated by antiserum to HDAC3 (histone deacetylase).
Among the consequences of this interaction are stabilization of the
co-repressor molecules and transcriptional silencing.

[0045] C21 was isolated in the laboratory of the present
inventor as a 201 bp fragment in a cDNA library prepared from

10   a bone marrow preparation highly enriched for human
hematopoietic stem cells. This fragment, which had no
homology with any sequence in the Gene Bank, hybridized with
RNA from K562 (a human erythroleukemia cell line) and a
placental library, and cDNAs prepared from these tissues were

15   used as a source of mRNAs for subsequent analysis. 5' RACE
identified an open reading frame (ORF) encoding a putative 514
amino acid protein. The nucleotide sequences and the putative
protein translation of these messages and the homologous mouse
sequences are presented in the Sequence Listing as SEQ ID NO:1

20   (human C21α cDNA), SEQ ID NO:2 (human C21α protein), SEQ ID
NO:3 (human C21β cDNA), SEQ ID NO:4 (human C21β protein), SEQ
ID NO:11 (mouse C21 cDNA), and SEQ ID NO:12 (mouse C21
protein). 3' RACE using a primer encoding nucleotides no.
1410-1425 in the ORF (SEQ ID NO:1) produced a new sequence

that diverged from the original sequence at nucleotide no.
1570. The new sequence encodes 45 amino acids, is in-frame,
and encodes an alternative carboxyl terminus for the protein.
5' RACE extended the published sequence by 80 nucleotides and
also revealed an additional mRNA with a small insert between
exons 2 and 3 of the original structure. This insert of 22
nucleotides GTAAGACTCTCCAACTCCCAAT (SEQ ID NO:16) is within the
5' UTR and is not present in all samples but occurs with a
frequency large enough to rule out its being a PCR artifact.
It may have a regulatory function or be involved in tissue
specificity.

[0046] Dot blots and Northern analysis showed that the
product of the C21 gene is expressed widely and Northern
analysis, using sequences from the ORF as a probe, revealed
size heterogeneity of the expressed mRNA. Northern analysis
with RNA extracted from a variety of sources including the
human leukemia cells K562, CHRF, Jurkat and Raji, showed two
predominant forms (4.7 kb and ~7.3 kb) of mRNA. The smaller
form is more abundant in hematopoietic tissues. Several
isoforms of the protein exist. C21β, differs from the
original sequence at the carboxyl-terminus (3' end of the
ORF). Western blotting with highly specific anti-peptide
antibodies shows several additional isoforms, whose pattern of
expression varies from tissue to tissue. The mouse homologue

has also been identified and Northern blotting of mouse

tissues shows size heterogeneity that is similar to the human

RNA. Analysis of the genomic DNA encoding C21 revealed that

the isoforms are produced by the use of alternative splice

5   donor sites. C21 maps to chromosome 3q26-27.

[0047] The cDNAs for C21 encode previously unknown members

of the β-transducin or WD-repeat family (Neer et al, 1994).

This family of proteins is defined by the occurrence of 4-8

repetitions of a conserved motif in each member polypeptide.

10   The conserved core of the repeating unit usually ends with the

sequence Trp-Asp, thus defining the WD40 repeat. Asparagine

(N) is almost as common as aspartic acid in the terminal

position. The WD-repeat motif is present in a large group of

functionally diverse proteins (>90 members). None are known

15   to have enzymatic activity. It is believed that the WD-repeat

domains mediate protein-protein interactions.   The splice

site that distinguishes between C21α and C21β is immediately

3' of the last WD-repeat sequence signature.

[0048] At the nucleotide level the sequence of C21α has 79%

20   homology to *Homo sapiens* mRNA for transducin (β)-1 like

protein (TBL1) (#Y12781, GenBank Accession No. NM005647). The

only homology of C21 and TBL1 to the prototypic signal

transducing guanine nucleotide binding regulatory (G) protein

β sub unit is in the WD-repeat domains. TBL1 has been mapped

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to the X chromosome and deletions in the region containing

TBL1 are associated with adult onset sensorineural deafness

(Bassi et al, 1999). Both C21 and TBL1 are homologous to a

Drosophila protein called *ebi* (GenBank Accession No. AF146345

5   for encoding nucleotide sequence). *Ebi* has been reported to

regulate epidermal growth factor receptor signaling (Dong et

al, 1999), promote the degradation of a repressor of neuronal

differentiation (Ttk88), and to limit S phase entry (Boulton

et al, 2000). The regions of maximal nucleotide conservation

10   among TBL1, *ebi* and C21 are in the N-terminal end of the

molecule and in the WD40 repeats. The C-terminal ends of

these proteins are not homologous. The carboxyl terminal exon

of C21α has some homology to several members of the Arp2/3

complex of proteins that control actin polymerization (Welch

15   et al, 1997). The equivalent region of C21β has no homology

to any known protein. Like TBL1, C21 contains a series of WD-

repeat domains within the C-terminal half of the ORF. The N-

terminal half of TBL1 interacts with both HDAC3 and SMRT to

form an effective transcriptional repressor (Guenther et al,

20   2000). Ectopic expression of TBL1 potentiates repression by

unliganded T3R. TBL1 does not bind directly to T3R; it binds

to SMRT and/or N-CoR, and this interaction was reported to

contribute an autonomous repression function to the complex.

The methods used in these experiments could not distinguish

among the possibilities that: 1) TBL1 activated N-CoR, producing increased repression, 2) it increased the quantity of N-CoR present in the target cells by protecting it from degradation or 3) it recruited an additional co-repressor.

5   [0049] C21, TBL1 and *ebi* all contain a variant F-box near the N terminus of the molecule. F-box proteins are a family of eukaryotic proteins that contain a ~40-amino acid motif called the F-box because it was first identified in cyclin F. F-box proteins, in combination with *Skp*1 and Cullin, forms

10   part of a E3 ubiquitin ligase that plays a critical role in the targeting of phosphorylated proteins for ubiquitination and subsequent proteasomal degradation. The bulk of this protein degradation is carried out by the 26S proteasome, and proteins are targeted to this compartment by the covalent

15   attachment of a multiubiquitin chain. Because proteolysis is irreversible, proteasomal degradation provides a unidirectional regulatory switch. The initiation of DNA replication, chromosome segregation, and exit from mitosis are all triggered by the destruction of key regulatory proteins

20   (DeSalle et al, 2001; Schwob et al, 1994; Glotzer et al, 1991). Ubiquitination is initiated by a ubiquitin-activating enzyme (E1), which adenylates ubiquitin and becomes linked to it via a thioester bond. Ubiquitin then is transferred to a ubiquitin-conjugating enzyme, E2. While E2s can attach

ubiquitin directly to lysine residues in a substrate, most physiological ubiquitination reactions probably require a ubiquitin ligase, or E3 (Hershko et al, 1983). E3s have been implicated in substrate recognition. Once the substrate is

5   multi-ubiquitinated, it then is recognized and degraded by the 26S proteasome (Hershko et al, 1983).

[0050] This pathway leading to proteasomal degradation has been reviewed recently (Ciechanover, 1993; Hochstrasser, 1992) and the role of the F-box has been reviewed by Pagano

10   (Kipreos et al, 2000). The F-boxes in C21, TBL1 and *ebi* all lack a tryptophan near the NH2-end of the motif that is associated with *Skp*1 binding to F-box proteins that recognize phosphorylated protein (Kipreos et al, 2000). A cDNA, described only as the "human homologue of *ebi*" encodes a

15   protein that the authors believe plays a role in the ubiquitin mediated destruction of β-catenin (Matsuzawa et al, 2001) and they suggested that this *ebi* can bind *Skp*1 and is a key component in a new pathway for targeting unphosphorylated proteins for ubiquitination and subsequent proteasomal

20   degradation (Matsuzawa et al, 2001; Liu et al, 2001).

[0051] Co-transfection of C21 with SMRT has been shown to lead to greatly enhanced SMRT expression in a transient expression model. Since SMRT is degraded via a ubiquitin-mediated mechanism and inhibition of ubiquitination leads to

elevation of SMRT, the data from the laboratory of the present

inventor suggest the possibility that C21 may act by

preventing the degradation of nuclear co-repressors. This

stabilization appears to be part of a complex regulatory

5   network that governs the level of unliganded nuclear receptor.

Although this result is the opposite of what might have been

predicted on the basis of the results described above for *ebi*-

mediated targeting of β-catenin (Matsuzawa et al, 2001), both

sets of data suggest that the C21 family interacts with

10   proteins subject to proteasomal degradation and the actual

outcome, protection or targeting, may depend on either the

nature of the target protein or the identity of the other

proteins that interact with C21. It may also be controlled by

which of the C21 family members that does the interacting.

15   The physiological effects of altering co-repressor stability

are not known but it is predicted that this family plays a

role in maintaining stem cells in their undifferentiated

state.

[0052] When the cDNA encoding C21α was over-expressed in

20   3T3 cells, the growth properties were altered: clones

expressing C21, grown under sub-optimal growth conditions,

plated with 2-3 times the efficiency of untransfected 3T3

cells. The increased plating efficiency is a result of

increased resistance to apoptotic stimuli leading to increased

survival in the interval before the cells attach to the plastic surface of the culture vessel. All cell lines that over-express this protein become resistant to the apoptotic effects of serum starvation. Apoptosis in thymocytes is

5 regulated by the activity of an NHR family member, RORγ (Kurebayashi et al, 2000). When this transcription factor is present, thymocyte development proceeds normally. However, in its absence, apoptosis increases and thymocyte development fails. Stabilization of a NHR-co-repressor complex by C21

10 could explain how C21 inhibits apoptosis.

[0053] Transgenic mice that over-express C21 in their hematopoietic cells have significant abnormalities in granulocyte development. As shown in Table 1 of Example 2, mice expressing C21 under the control of the vav promoter,

15 have normal numbers of total WBC but have a significant "shift to the left", i.e., these animals have very few mature granulocytes and an increased number of immature forms. This phenotype resembles what happens when wild type RARα is over-expressed in hematopoietic cells (Kastner et al, 2001)

20 suggesting that anything that leads to an increase in the proportion of unliganded RAR inhibits RA-controlled differentiation.

[0054] Based on the above characterization of C21, the present invention is directed to a C21 polypeptide having the

amino acid sequence of SEQ ID NO:2 (human C21α) which

interacts with, and has the activity of modulating the

stability of, transcriptional regulatory complexes (i.e.,

transcriptional co-repressor complexes) that regulate nuclear

5   hormone receptor activity and to a nucleic acid molecule

encoding the polypeptide of the present invention. As will be

appreciated by those of skill in the art, the polypeptide of

the present invention is also intended to encompass a fragment

of the C21 polypeptide which retains the activity of the full-

10   length C21 polypeptide. Further comprehended by the C21

polypeptide according to the present invention is a variant of

the c21 polypeptide having an amino acid sequence with at

least 85%, preferably at least 90% or 95%, sequence identity

to SEQ ID NO:2, which interacts with, and also has the

15   activity of modulating the stability of, transcriptional

regulatory complexes that regulate nuclear hormone receptor

activity. For instance, the human β isoform of C21 (SEQ ID

NO:4) and the mouse C21 (SEQ ID NO:12), which have

approximately 92% and 97% sequence identity with SEQ ID NO:2

20   (human C21α), are considered as variant polypeptides

comprehended by the polypeptide of the present invention.

[0055] A second aspect of the present invention provides

for antibodies raised against the polypeptide according to the

- 30 -

present invention and molecules which includes the antigen-binding portion of such antibodies.

[0056] It should be understood that when the term "antibodies" is used with respect to the antibody embodiments of the present invention, this is intended to include intact antibodies, such as polyclonal antibodies or monoclonal antibodies (mAbs), as well as proteolytic fragments thereof such as the Fab or F(ab')$_2$ fragments. Furthermore, the DNA encoding the variable region of the antibody can be inserted into other antibodies to produce chimeric antibodies (see, for example, U.S. Patent 4,816,567) or into T-cell receptors to produce T-cells with the same broad specificity (see Eshhar, et al, 1990 and Gross et al, 1989). Single-chain antibodies can also be produced and used. Single-chain antibodies can be single-chain composite polypeptides having antigen binding capabilities and comprising a pair of amino acid sequences homologous or analogous to the variable regions of an immunoglobulin light and heavy chain (linked V_H-V_L or single-chain F_V). Both V_H and V_L may copy natural monoclonal antibody sequences or one or both of the chains may comprise a CDR-FR construct of the type described in U.S. Patent 5,091,513 (the entire content of which is hereby incorporated herein by reference). The separate polypeptides analogous to the variable regions of the light and heavy chains are held

together by a polypeptide linker. Methods of production of

such single-chain antibodies, particularly where the DNA

encoding the polypeptide structures of the V_H and V_L chains are

known, may be accomplished in accordance with the methods

5   described, for example, in U.S. Patents 4,946,778, 5,091,513

and 5,096,815, the entire contents of each of which are hereby

incorporated herein by reference.

[0057] An antibody is said to be "capable of binding" a

molecule if it is capable of specifically reacting with the

10   molecule to thereby bind the molecule to the antibody. The

term "epitope" is meant to refer to that portion of any

molecule capable of being bound by an antibody which can also

be recognized by that antibody. Epitopes or "antigenic

determinants" usually consist of chemically active surface

15   groupings of molecules such as amino acids or sugar side

chains and have specific three dimensional structural

characteristics as well as specific charge characteristics.

[0058] Polyclonal antibodies are heterogeneous populations

of antibody molecules derived from the sera of animals

20   immunized with an antigen.

[0059] Monoclonal antibodies (mAbs) are a substantially

homogeneous population of antibodies to specific antigens.

MAbs may be obtained by methods known to those skilled in the

art. See, for example Kohler et al, (1975); U.S. Patent No.

4,376,110; Harlow et al, (1988); and Colligan et al, (2001),
the entire contents of which references are incorporated
entirely herein by reference. Such antibodies may be of any
immunoglobulin class including IgG, IgM, IgE, IgA, and any
subclass thereof. The hybridoma producing the mAbs of this
invention may be cultivated in vitro or in vivo. High titers
of mAbs can be obtained by in vivo production where cells from
the individual hybridomas are injected intraperitoneally into
pristane-primed Balb/c mice to produce ascites fluid
containing high concentrations of the desired mAbs. MAbs of
isotype IgM or IgG may be purified from such ascites fluids,
or from culture supernatants, using column chromatography
methods well known to those of skill in the art.

[0060] Chimeric antibodies are molecules, the different
portions of which are derived from different animal species,
such as those having a variable region derived from a murine
mAb and a human immunoglobulin constant region. Chimeric
antibodies are primarily used to reduce immunogenicity during
application and to increase yields in production, for example,
where murine mAbs have higher yields from hybridomas but
higher immunogenicity in humans, such that human/murine
chimeric or humanized mAbs are used. Chimeric and humanized
antibodies and methods for their production are well-known in
the art, such as Cabilly et al (1984), Morrison et al (1984),

Boulianne et al (1984), Cabilly et al European Patent 0 125 023 (1984), Neuberger et al (1985), Taniguchi et al European Patent 0 171 496 (1985), Morrison et al European Patent 0 173 494 (1986), Neuberger et al WO 8601533 (1986), Kudo et al

5 European Patent 0 184 187 (1986), Sahagan et al (1986); Robinson et al WO 9702671 (1987), Liu et al (1987), Sun et al (1987), Better et al (1988), and Harlow et al (1988). These references are hereby incorporated herein by reference.

[0061] A "molecule which includes the antigen-binding

10 portion of an antibody," is intended to include not only intact immunoglobulin molecules of any isotype and generated by any animal cell line or microorganism, or generated in vitro, such as by phage display technology for constructing recombinant antibodies, but also the antigen-binding reactive

15 fraction thereof, including, but not limited to, the Fab fragment, the Fab' fragment, the $F(ab')_2$ fragment, the variable portion of the heavy and/or light chains thereof, and chimeric or single-chain antibodies incorporating such reactive fraction, or molecules developed to deliver therapeutic

20 moieties by means of a portion of the molecule containing such a reactive fraction. Such molecules may be provided by any known technique, including, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques.

[0062] A further aspect of the present invention is directed to a nucleic acid molecule which encodes the polypeptide of the present invention. As one embodiment, the nucleic acid molecule contains the nucleotide sequence encoding the amino acid sequence of SEQ ID NO:2. In a preferred embodiment, the nucleotide sequence encoding SEQ ID NO:2 (human C21α) is nucleotides 161 to 1705 of SEQ ID NO:1.

[0063] The nucleic acid molecule of the present invention further comprehends those nucleic acid molecules that encode variants of the polypeptide of SEQ ID NO:2, such as the variant polypeptides of SEQ ID NO:4 or SEQ ID NO:12. These nucleic acid molecules have high homology to nucleotides 161 to 1705 of SEQ ID NO:1 and include the nucleic acid molecules having the nucleotide sequence of nucleotides 161 to 1705 of SEQ ID NO:3 or nucleotides 202 to 1746 of SEQ ID NO:11, which hybridize to nucleotides 161 to 1705 of SEQ ID NO:1 under highly stringent conditions.

[0064] The nucleotide sequence of naturally-occurring variants of human C21α in question, such as, for example, allelic variations and splice variants, may be determined by hybridization of a cDNA library using a probe which is based on the identified polynucleotide, under highly stringent conditions. Stringency conditions are a function of the temperature used in the hybridization experiment and washes,

the molarity of the monovalent cations in the hybridization

solution and in the wash solution(s) and the percentage of

formamide in the hybridization solution. In general,

sensitivity by hybridization with a probe is affected by the

5 amount and specific activity of the probe, the amount of the

target nucleic acid, the detectability of the label, the rate

of hybridization, and the duration of the hybridization. The

hybridization rate is maximized at a Ti (incubation

temperature) of 20-25°C below Tm for DNA:DNA hybrids and 10-

10 15°C below Tm for DNA:RNA hybrids. It is also maximized by an

ionic strength of about 1.5M Na$^+$. The rate is directly

proportional to duplex length and inversely proportional to

the degree of mismatching.

[0065] Specificity in hybridization, however, is a function

15 of the difference in stability between the desired hybrid and

"background" hybrids. Hybrid stability is a function of

duplex length, base composition, ionic strength, mismatching,

and destabilizing agents (if any).

[0066] The Tm of a perfect hybrid may be estimated for

20 DNA:DNA hybrids using the equation of Meinkoth et al (1984),

as

$$Tm = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% form) - 500/L$$

and for DNA:RNA hybrids, as

25 $$Tm = 79.8°C + 18.5 (\log M) + 0.58 (\%GC) -$$

$$11.8 \ (\%GC)^2 - 0.56(\% \ form) - 820/L$$

where

M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-

75%,

% form, percentage formamide in hybridization

solution, and

L, length hybrid in base pairs.

[0067] Tm is reduced by 0.5-1.5°C (an average of 1°C can be used for ease of calculation) for each 1% mismatching.

[0068] The Tm may also be determined experimentally. As increasing length of the hybrid (L) in the above equations increases the Tm and enhances stability, the full-length human C21α DNA sequence can be used as the probe.

[0069] Filter hybridization is typically carried out at 68°C, and at high ionic strength (e.g., 5 - 6 X SSC), which is non-stringent, and followed by one or more washes of increasing stringency, the last one being of the ultimately desired stringency. The equations for Tm can be used to estimate the appropriate Ti for the final wash, or the Tm of the perfect duplex can be determined experimentally and Ti then adjusted accordingly.

[0070] Hybridization conditions should be chosen so as to permit allelic variations and splice variants, but avoid

hybridizing to other genes. In general, stringent conditions
are considered to be a Ti of 5°C below the Tm of a perfect
duplex, and a 1% divergence corresponds to a 0.5-1.5°C
reduction in Tm. Typically, rat clones were 95-100% identical

5   to database rat sequences, and the observed sequence
divergence may be artifactual (sequencing error) or real
(allelic variation). Hence, use of a Ti of 5-15°C below, more
preferably 5-10°C below, the Tm of the double stranded form of
the probe is recommended for probing a cDNA library.

10 [0071] As used herein, highly stringent conditions are
those which are tolerant of up to about 15% sequence
divergence. Without limitation, examples of highly stringent
(5-15°C below the calculated Tm of the hybrid) conditions use
a wash solution of 0.1 X SSC (standard saline citrate) and

15 0.5% SDS at the appropriate Ti below the calculated Tm of the
hybrid. The ultimate stringency of the conditions is
primarily due to the washing conditions, particularly if the
hybridization conditions used are those which allow less
stable hybrids to form along with stable hybrids. The wash

20 conditions at higher stringency then remove the less stable
hybrids. A common hybridization condition that can be used
with the highly stringent wash conditions described above is
hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X

Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured, fragmented

salmon sperm DNA at an appropriate incubation temperature Ti.

[0072] Other additional aspects of the present invention

include a vector containing the nucleic acid molecule of the

present invention, a host cell transformed with the nucleic

acid of the present invention, and a method for producing the

polypeptide of the present invention. This method involves

culturing the host cell transformed with the nucleic acid

molecule of the present invention in nutrient medium and then

recovering the polypeptide expressed and produced by the

cultured host cell.

[0073] The C21 family of regulatory proteins which is

differentially expressed by early hematopoietic cells has been

identified by the present inventor. These proteins, referred

to herein as polypeptides of the present invention, interact

with transcriptional repressor complex and are members of a

new class of regulatory molecules that regulate the stability

of their targets by controlling their susceptibility to

ubiquitin mediated proteasomal degradation. Evidence is

accumulating that the regulation of transcriptional activity

is a central mechanism in the control of lineage specification

and that members of the C21 family of proteins play an

important role in this process. C21 exists in multiple

isoforms, and two of these (α and β) are expressed at high

levels in immature hematopoietic cells. Expression of both C21 α and β isoforms declines during hematopoietic maturation but the β form is found in some mature myeloid cells.

[0074] C21 interacts with the transcriptional repressor complex and specifically binds to SMRT. This binding results in elevation of the intracellular concentration of SMRT. Aberrant or unregulated alterations in C21 levels may have leukemogenic potential. The laboratory of the present inventor has already shown that over-expression leads to abnormal myeloid development in young mice, and these animals will be followed as they age. Approximately 40% of acute myelogenous leukemia (AML) cases of the M2 subtype are due to a chromosomal translocation that combines a sequence-specific DNA binding protein, AML1, with a potent transcriptional repressor, ETO. Like C21, ETO interacts with nuclear receptor co-repressors SMRT and N-CoR, which recruit histone deacetylase (HDAC) to the AML1-ETO oncoprotein (Muramatsu et al, 2001; He et al, 1998). Inhibitors of HDAC are the potent inducer/enhancers of differentiation in acute myeloid leukemia (Kosugi et al, 1999) and the potential therapeutic benefit of HDAC inhibition has been established by the use of enzyme inhibitors *in vitro* and at least one reported case of experimental therapy (Kramer et al, 2001). CI-994 (acetyldinaline), an HDAC inhibitor, is currently in Phase II

trials. If members of the C21 family regulate HDAC recruitment by N-CoR/SMRT, they may provide another target for therapeutic attack in this disease.

[0075] The gene encoding C21 is located in a chromosomal region that is known to be the site of many chromosomal abnormalities. A significant proportion of AML, of the non-M2 type, involve translocations that involve the q arm of chromosome 3 and these have a poor prognosis (Grimwade et al, 1998). Interestingly, leukemias involving chromosome 3q are among the subset of malignancies that do not have mutations in p53 (Munoz et al, 1999). Recently the observation was made that AML with chromosome 3q abnormalities was highly correlated with prior exposure to mutagens (Lindquist et al, 2000).

[0076] The consequences of the interaction of C21 with the nuclear transcription repressor complex have only begun to be explored, but several clinically important consequences suggest themselves in addition to a possible involvement of C21 with leukemia. The stabilizing interaction of C21 with nuclear co-repressors may be replicated by small molecules that mimic C21 binding. Such molecules could provide a new and novel means of altering the differentiation of cultured hematopoietic cells and might allow the expansion of undifferentiated progenitors or stem cells (Muramatsu et al).

[0077] The anti-apoptotic effect of over-expression could protect stem cells (hematopoietic as well as others) from inappropriate or excessive apoptotic stimuli. Defects in the related TBL-1 gene lead to degeneration of the Organ of Corti in middle age, perhaps suggesting that this gene family plays a role in protection against environmental or age-related damage. If means were available to control the expression of C21 in specific tissues or to replicate their effects with small molecules, it might extend their functional life *in vivo* and also permit their growth in culture. Selective expansion of normal HSC could provide a source of uncontaminated stem cells for autologous BM transplants in patients with both hematological malignancies and advanced solid tumors as well as providing a large pool of cells that could be used as vehicles for genetic therapies. Other proteins with anti apoptotic effects such as Bcl-2, prevent the elimination of cells that are otherwise targeted for destruction, and act as oncogenes. The high frequency of genetic abnormalities at or near 3q26 may indicate the C21 also can serve as an oncogene. Amplification or over-expression of C21 might reduce the ability of cells to respond to environmental changes and thus might alter resistance to cytotoxic drugs. Pharmacological manipulation of C21 levels could be useful in altering

resistance to chemotherapeutic agents or after toxic or radioactive exposures.

[0078] Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLE 1

[0079] Using CD34+/CD38- cells as starting material, the laboratory of the present inventor has identified four mRNAs, expressed by these cells that are either absent or present in reduced amounts in more mature CD34+/CD38+ cells. One of these cDNAs (C40) encodes a known member of the subfamily of protein phosphatases (CL100) that exhibits dual substrate specificity for phosphotyrosine- and phosphoserine/threonine-containing substrates and specifically inactivates MAP kinases. This phosphatase has been shown to play a role in regulating the differentiation of several cell types. The second cDNA, (C23) is identical to LR11 (gp250) a member of the Low Density Lipoprotein Receptor family. LR11 is unusual in that in addition to 11 ligand-binding repeats, it contains a series of fibronectin type III repeats near its carboxyl-terminal end which are similar to those found in cytokine receptors. It is highly expressed in developing brain but hematopoietic expression has not been reported. The 178 bp

fragment that was originally cloned is part of a 4145 bp 3'
UTR that had not been previously sequenced and is among the
largest human 3' UTR ever reported. The other isolates (C21
and C12) do not correspond to known protein sequences. They
are homologous to EST sequences from a fetal brain library.
C21 encodes a previously unknown gene that is a member of the
WD-40 family. An open reading frame encoding a 515 amino acid
protein has been identified.

MATERIALS AND METHODS

Cell Preparations

[0080] Bone marrow specimens were obtained from discarded
orthopedic surgical specimens. Peripheral blood leukocytes
were obtained from 20 ml whole blood samples provided by
volunteers. CD34+/CD38- cells were prepared from adult marrow
by a combination of immunomagnetic techniques and cell
sorting. Both positive and negative immunoselection were
used. Negative selection to enrich CD34+ cells was performed
using an immunomagnetic system (STEMSEPT™; Stem Cell
Technologies, Vancouver, British Columbia, Canada). The
system uses a cocktail of monoclonal antibodies that have been
bound in bispecific antibody complexes directed against cell
surface antigens on human hematopoietic cells (CD2, CD3, CD14,
CD19, CD25, CD56, CD66b and glycophorin A) and dextran. The
separation column consists of dextran-coated ferro-magnetic

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steel wool packed in a plastic column and mounted in a high

gauss magnetic field. The column retains antibody-coated

cells. Since the magnetic separation used in the first step

of this preparation removes cells expressing lineage specific

5   markers, the resultant cell populations are all lineage

negative (Lin-). The Lin-CD34-enriched cells were then

stained with anti-CD34-PE (clone HPCA-1; Becton Dickinson, San

Jose, CA) and anti-CD38 FITC (clone HIT2; CALTAG, South San

Francisco, CA). The antibodies were used at saturating

10   concentrations, approximately 0.5 ug/ml. The stained cells

were sorted on an Epics Elite modified for high speed sorting.

CD34+/CD38+ and CD34+/CD38-populations were sorted. A

representative result obtained using this protocol is shown in

Figures 1A-1D. The final purity of the sorted populations was

15   always >98%.

[0081] Peripheral blood neutrophils, lymphocytes and

monocytes were also prepared by sorting. Whole blood was

incubated in Tris-buffered ammonium chloride to lyse the

erythrocytes. The white blood cells (WBC) were pelleted by

20   low speed centrifugation and resuspended in phosphate buffered

isotonic saline containing 1 mg/ml of bovine serum albumin

(PBS-BSA). The unstained samples were sorted on the basis of

low angle (forward) and right angle light scatter and three

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populations, corresponding to lymphocytes, monocytes and neutrophils were isolated.

Construction of Subtracted Library

[0082] Total RNA was prepared from the sorted cells and double-stranded cDNAs were prepared from these RNAs using the CapFinder™ PCR cDNA Synthesis Kit provided by Clontech Laboratories (Palo Alto, CA). cDNAs from CD34+/CD38- and CD34+/CD38+ cells were used as "tester" and "driver" to produce the subtracted cDNA. A total of 57,000 CD34+/CD38- cells were used to prepare the tester RNA while 478,000 CD34+/CD38+ cells were used to prepare the driver RNA. The RNA was isolated using a modification of the method of Chomczynski et al (1987) (Stratagene, La Jolla, CA). The tester and driver cDNAs were digested with Rsa I, a four base cutter that leaves blunt ends. The tester cDNA was then divided into two portions, each of which was ligated to the adaptors provided by Clontech in their PCR Subtraction kit. These adaptors lack terminal PO_4 and thus only a single adaptor was added to each tester strand. Tester cDNA and driver cDNAs were heated to 100°C, for 3 minutes, and then the driver hybridized with each of the tester samples at 68°C, for 8 hours. Under these conditions, abundant messages hybridize rapidly while rare messages anneal more slowly and after hybridization, the residual single stranded material is

enriched for low abundance sequences. The two hybridization
mixtures (one for each of the adaptors) were mixed without
being denatured again. Additional denatured driver was added
and the mixture hybridized for 16 hours. Differentially
expressed cDNAs were then amplified by PCR. The missing
complementary strands of the adaptors were filled in by a
brief incubation at 75°C, and then thermally denatured to
begin a conventional PCR cycle. After 30 cycles the reaction
was stopped and a small aliquot removed, diluted and amplified
further using the nested primers provided in the kit. The
pCR2.1 (plasmid) vector was used to prepare the library. As
supplied by Invitrogen (Carlsbad, CA), pCR2.1 contains a pre-
nicked insertion site for T/A cloning.

3' and 5' RACE

[0083] 3' and 5' RACE were performed with Marathon-ready
cDNA (Clontech) according to the manufacturer's protocol. The
first PCR was performed with a specific primer and an adaptor
primer (primer 1) using the following conditions: 1 minute at
94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds
at 60°C, and 2-4 minutes at 72°C with a final 10 minute
extension at 72°C. The PCR products were diluted and
subjected to a second PCR using an internal specific primer
and adaptor primer 2. After electrophoresis in agarose
containing ethidium bromide, the bands were excised. The DNA

was purified using a silica-gel membrane (Gel Purification

Kit; Qiagen, Valencia, CA) and ligated into PCR2.1 vector.

PCR using specific primers was used to identify positive

colonies. Five plasmids from each band were sequenced.

5   *Library Screening*

[0084] The subtracted cDNA library was used to transform *E.*

coli InV2F' cells (Invitrogen). Plasmids were prepared from

individual colonies. After digestion with EcoRl, the plasmid

DNAs were electrophoresed through a 1% agarose gel and

10   transferred to nitrocellulose membranes. Duplicate membranes

were hybridized with ^{32}P-labeled cDNA from CD34+/CD38- and

CD34+/CD38+ cells. The membranes were hybridized at 42°C

overnight and washed under high stringency conditions. X-ray

film, exposed at -70°C overnight with an intensifier screen,

15   was used to detect the hybridized DNA. Plasmids, which

hybridized strongly with the ^{32}P-labeled CD34+/CD38- cDNA but

not with the ^{32}P-labeled CD34+/CD38+ cDNA, were sequenced.

"Virtual" Northern Blots

[0085] Double stranded cDNAs synthesized from a new

20   preparation of Lin-, CD34+/CD38- (39,000 cells) and Lin-,

CD34+/CD38+ (410,00 cells) were electrophoresed on 1% agarose

gel and transferred to nitrocellulose membranes. Five

replicate membranes were prepared and these were hybridized

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with the probes prepared from each of the cDNAs that appeared
to be differentially expressed on the basis of the screening
assay described above.

Northern Analysis and Dot Blots

[0086] Multiple Tissue Northern Blot™ and Human RNA Master
Blot™ membranes were purchased from Clontech. Hybridizations
were performed with (α-^{32}P)dATP labeled probes that also
contained a modified dCTP that facilitates removal of the
hybridized probe so that the blot can be hybridized repeatedly
(Strip-ez™, Ambion Inc, Austin TX). The Master Blots, as
provided by the manufacturer, include 16 RNAs from regions of
the central nervous system. Results are only shown from whole
brain, medulla and spinal cord. The images obtained after
scanning the autoradiographs of the blots from these tissues
(samples D6, D7 and D8 of Figures 3B-3D) were moved to the
positions shown in the figure.

Ribonuclease Protection Assay

[0087] ^{32}P-UTP labeled probes were transcribed using T7 RNA
polymerase from double stranded DNA templates. For
hybridization, 1 ug of total RNA was dried, dissolved in
hybridization buffer and mixed with the labeled probe (600,000
cpm/sample). The samples were denatured at 90°C and then
hybridized at 56°C overnight. The unhybridized RNA was

digested with a mixture of RNases A and T1. After

hybridization and RNase digestion the samples were treated

with proteinase K, extracted with phenol, ethanol precipitated

and dried. They were analyzed by electrophoresis in a

5   standard acrylamide sequencing gel and the protected sequences

were detected by autoradiography. The C23 probe was prepared

by amplifying a 150 bp cDNA from original isolate.

Sense Primer   5'-AGGGAATGTAACCCTTCTCA-3' (SEQ ID NO:5)

Anti-Sense Primer 5'-TCTTACTAGATGCAGTGACC-3' (SEQ ID NO:6)

10   [0088] The resultant cDNA was then unidirectionally ligated

into pCR3.1 TA vector and linearized with EcoRl. A ribosomal

18s probe (pTRI RNA18s), purchased from Ambion, was used to

normalize the quantity of material applied to each lane of the

gel. The protected fragment is 80 bp long.

15   *RT-PCR*

[0089] The expression of C21 and C12 mRNA by human leukemic

cell lines was measured by RT-PCR. The RNAs were reverse

transcribed with MMLV-RT using oligo (dT) to prime the

reaction. For C21, the full 1548 bp ORF was used as a

20   template in the subsequent PCR, while for C12 a 500 bp

fragment was used. The primers used were:

C21   sense   GATGAGTATAAGCAGTGATGT (SEQ ID NO:7)

       antisense   CTATTTTTGTTCTTTCCGAAGGTCTAATA (SEQ ID NO:8)

C12   sense   CAACAGAGCTTCACTTTACCC (SEQ ID NO:9)

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antisense   CTAGGGATGGTTTCCATGA (SEQ ID NO:10)

[0090] The Both PCR reactions were performed using the
following conditions: 1 minute at 94°C followed by 30 cycles
of 30 seconds at 94°C, 30 seconds at 58°C, and 2 minutes at

5   72°C. The PCR products were diluted and electrophoresed in
agarose containing ethidium bromide.

Sequence Analysis

[0091] Plasmid DNA was sequenced by the chain termination
method in the Core Sequencing Facility of Kaplan Cancer Center

10   using an Applied Biosystems automated sequencer. The
resulting sequences were analyzed using software provided by
Research Computing Resources of the Kaplan Comprehensive
Cancer Center.

RESULTS

15   [0092] Lin-/CD34+/CD38- and Lin-/CD34+/CD38+ cells were
purified as described in the Materials and Methods section and
used to prepare the cDNA library. The subtracted, normalized
PCR-amplified difference product was TA cloned into pCR2.1 to
produce a plasmid library. The library was plated, and

20   individual colonies isolated and expanded. Forty insert-
containing clones were analyzed. Four clones were obtained
that reacted predominantly with the tester cDNA and probes
were synthesized from these. Figure 2A shows "virtual"

Northern blots produced by hybridizing these probes with cDNA

produced from Lin- CD34+/CD38- and CD34+/CD38+ BMC. Figure 2B

shows the results of RT-PCR to amplify defined sequences from

each of the four messages, under conditions in which the

5   amount of product produced is proportional to the quantity of

message present. The mRNA for CD38 was also amplified to

provide a non-serological indication of the purity of the Lin,

CD34+/CD38- cells.

[0093] Clone C40 contained a 352 bp DNA fragment. Its

10   sequence is identical to nucleotides 1192-1538 of the protein

phosphatase CL100 (EMB#X68277). In view of this large region

of sequence identity, it was concluded that the CL100 was the

differentially expressed message identified by C40 and no

further analysis was attempted. The other three isolates did

15   not correspond to known protein sequences. All of them were

homologous to EST sequences present in public domain sequence

databases.

[0094] C23 was originally isolated as a 178 bp fragment

that was identical to nucleotides 804-981 of a 1896 bp

20   sequence obtained from a fetal brain library (#U90916). It is

also homologous to several smaller EST sequences. No protein

homology was detected. A probe, prepared from C23, hybridized

with RNA from human spleen and cDNA prepared from this splenic

RNA was used to sequence and identify the gene. Sequential 5'

and 3' RACE using the sequence of the original fragment to design primers, confirmed the EST sequence. In addition ~2 kb of new sequence was determined. No homology to any functional gene was found until >4000 bp was sequenced. Sequencing the 5' end of a 5' RACE product demonstrated that C23 is a member of the LDLR family. Subsequent sequencing indicated that it is identical to an unusual member of the LDLR family, LR11 (Yamazaki et al, 1997; Novak et al, 1996).

[0095] Dot blots with RNA from a wide range of human tissues show that C23 is widely expressed and is highly expressed in fetal tissues (Figure 3A). The highest level of expression was found in neural tissue, liver, pituitary and peripheral WBC. Significant expression was also detected in hematopoietic tumor cell lines such as CHRF, Raji, KG1, and U937 but not in K562 (data not shown). RNase protection analysis demonstrated that C23 was highly expressed in neutrophils and could be detected in lymphocytes (Figure 4A).

[0096] C21 was originally isolated as a 201 bp fragment. No protein homology was detected. This fragment was used to probe a placental cDNA library and a 2209 bp fragment was identified and sequenced. cDNA prepared from the K562 RNA was used to sequence the remainder of C21. Using both 5' and 3' RACE, a poly A region of this cDNA and a complete open reading frame were identified. The 3596 bp sequence is shown in

Figures 5A-5B, and the sequencing strategy used to characterize this gene is shown in Figure 6B. The 3'-UTR encoded in this ORF is >2000 bp. The cDNA encodes a previously unknown member of the WD-40 family (Neer et al, 1994). At the nucleotide level, the sequence has 79% identity across the open reading frame to *Homo sapiens* mRNA for transducin (beta)-like 1 (TBL1) (#Y12781) (Bassi et al, 1999) and 68% homology to *ebi,* a drosophila nuclear protein (Dong et al). Prosite analysis detected four WD-40 repeats within the ORF.

[0097] A dot blot (Figure 3B) shows that the product of this gene is expressed widely and the virtual Northern blot shown in Figure 2A indicates that it is preferentially expressed in the CD34+/CD38- population, and it appears to be a highly expressed gene. RT-PCR using primers that flanked the ORF, amplified a 1548 bp cDNA from mRNA templates extracted from the human leukemic cell lines CHRF, Jurkat, HL60, K562, U937 and Raji (Figure 4B). On Northern blots, a probe encoding the ORF, hybridized with 4.3 kb and ~7.8 kb messages in RNAs extracted from a variety of sources, including the human leukemic cells (data not shown).

[0098] C12 was originally isolated as a 267 bp fragment that is homologous to several ESTs. No protein homology was detected. A virtual Northern blot (Figure 2A) confirmed that

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this gene is differentially expressed in the CD34+/CD38-
population. It, too, has a relatively wide tissue expression
(Figure 3C) with the highest levels occurring in adult adrenal
gland, heart, stomach and liver. Among the hematopoietic

5   organs, both adult bone marrow and thymus had significant
levels. Using RNA from a Jurkat cell library, ~1 kb of cDNA
that appears to be the 3' end of the molecule was sequenced.
The laboratory of the present inventor has been unable to
obtain a sequence encoding an open reading frame using either

10   5' extension of the original sequence or by using this
sequence as a probe with a placental cDNA library. However,
since an RNase protection assay, using the original C12
isolate as a probe, protected the appropriately sized fragment
when hybridized with RNA isolated from K562 (data not shown)

15   and RT-PCR amplified a cDNA of the correct size from the panel
of human leukemic cells, it is expected that this is an
expressed message and not a cloning artifact.

DISCUSSION

[0099] The molecular processes that maintain the stem cell

20   pool are largely unknown. Understanding this process requires
identifying the unique attributes of PHSC. Because of their
rarity, molecular characterization of PHSC has been difficult
to achieve. Much of what has been accomplished has been
directed towards analyzing the temporal expression of genes

that are known to play a role in various aspects of hematopoiesis. Primary cultures of hematopoietic progenitors and developing embryonic stem (ES) cells express cytokine and growth factor receptors and transcription factors known to be important in hematopoiesis (Cheng et al, 1996; Keller et al, 1993; Orlic et al, 1995). These studies have been extended to the single cell level and expression by "sister" pairs of cells has been studied (Brady, 1993). Targeted deletion of several transcription factors known to be active in hematopoietic cells results in failure of either primary or definitive hematopoiesis (Zhang et al, 1998b; Simon, 1998; Meyer et al, 1998; Lessard et al, 1998; Neubauer et al, 1998; Okuda et al, 1998).

[00100]   cDNA libraries prepared from enriched stem cell preparations have been used to identify genes uniquely expressed by stem cells or single CD34+/CD38- cells. Tyrosine kinases and phosphatases that are differentially expressed in early hematopoietic cells (Hoehn et al, 1996; Bierhuizen et al, 1997; Dosil et al, 1996) have been found by using degenerate PCR. Tnk-, a tyrosine kinase gene, was isolated from CD34+/CD38- derived mRNA (Hoehn et al, 1996) and a similar strategy was used to identify FLP-1 (Fetal Liver Phosphatase (Dosil et al, 1996).

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[0100] The laboratory of the present inventor has extended the search for genes that are differentially expressed in PHSC by using PCR-driven subtraction and has identified four mRNAs that are differentially expressed by Lin-, CD34+/CD38- cells. None of these genes encode a message whose expression is limited to PHSC. All of them are expressed in a variety of tissues, suggesting that they are involved in processes that are important to many cell types and not just hematopoietic cells. To date, none of the efforts to identify a gene uniquely expressed by PHSC have been successful. Such hematopoietic, stem cell-specific regulatory genes may be very rare or perhaps do not exist. Stem cells, regardless of the function of their differentiated progeny, share many characteristics. They are self-renewing, but rarely actively proliferating; they are protected from toxic agents in their environment and finally, they are isolated from excessive stimulation by environmental factors that might otherwise deplete the stem cell pool. These properties are not unique requirements for cells of the hematopoietic lineage. Furthermore evidence has accumulated suggesting that the plasticity of stem cells is far greater than previously believed (Lemischka, 1999). Cells derived from muscle and brain have been shown to be capable of developing into hematopoietic cells (Jackson et al, 1999; Bjornson et al,

1999) and bone marrow derived cells have been shown to give rise to hepatocytes (Theise et al, 2000) and myoblasts (Gussoni et al, 1999). How the fate of any of these cells is determined remains unclear.

5   [0101] One of the genes that is shown to be differentially expressed in CD34+/CD38- cells, clone C40, is identical to CL100 (Alessi et al, 1993). In the bone marrow, CL100 is expressed by CD34+/CD38- cells, and it could not be detected in mature hematopoietic cells. It is a member of the
10 subfamily of protein phosphatases that exhibits dual substrate specificity for phosphotyrosine and phosphoserine/threonine residues in proteins and is related to the late H1 gene of vaccinia virus. The CL100 phosphatase, expressed and purified in bacteria, inactivates recombinant MAP kinase *in vitro* by
15 the concomitant dephosphorylation of both its phosphothreonine and phosphotyrosine residues. Deactivation of these signaling cascades at the MAPK level is critically dependent on dephosphorylation of the TXY motif by members of the MKP family of dual-specificity phosphatases.

20 [0102] Since MAP kinases play such a central role in the regulation of transcriptional activation, an enzyme activity that inactivates MAP kinase could serve to protect stem cells of any lineage from inadvertent or inappropriate activation. For example it has recently been shown that all-trans retinoic

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acid, which enhances the long-term hematopoietic repopulating
activity of cultured hematopoietic cells (Purton et al, 2000)
and delays the differentiation of PHSC (Purton et al, 1999)
increases the levels of the mouse orthologue of CL100 (MKP-1)

5   (Lee et al, 1999). MKP-1 also plays a role in maintaining
quiescence in smooth muscle cell and is down-regulated when
proliferation is induced (Lai et al, 1996). In rat arterial
smooth muscle cells over expressing MKP-1, growth was arrested
in the G1 phase and entry into the S phase was blocked.

10   Similarly, MKP-1 is constitutively expressed on myoblast C2C12
cells, but when these cells are transferred to differentiation
medium, expression declines (Bennett et al, 1997). These
results suggest that CL100 may play an important role in
maintaining stem cells in a quiescent state and protecting the

15   stem cell pool from depletion.

[0103] C23 is identical to LR11 (gp250), an unusual member
of the Low Density Lipoprotein Receptor family (Novak et al,
1996; Horn et al, 1997; Jacobsen et al, 1996); Yamazaki et al,
1996). This growing family of receptors transports

20   macromolecules into cells by receptor mediated endocytosis.
The family members bind a wide range of unrelated ligand and
it is not known if all of this binding is functionally
significant. LR11 is unusual in that in addition to 11
ligand-binding repeats, it contains a series of fibronectin

type III repeats near its carboxyl-terminal end which are similar to those found in cytokine receptors Yamazaki et al, 1997). It is highly expressed in brain but hematopoietic expression has not been reported. In the peripheral blood, neutrophils are the predominant source of mRNA for LR11. Among the striking properties of LR11 is its high degree of structural conservation (>80% identity among mammals). It is also unusual in that its expression is unaffected by cholesterol and estrogen. The 178 bp fragment that was cloned here is part of a 4145 bp 3' UTR that had not been previously sequenced. This is among the largest known human 3' UTRs Pesole et al, 1998).

[0104] C21 encodes a previously unknown gene that is a member of the WD-40 family (Neer et al, 1994). This mRNA is expressed widely but the most striking feature of our survey of the expression of this gene is the high level detected in fetal tissues (Figure 3B, row E). The WD-40 group is a large family of proteins, all of whom appear to be regulatory in function. It is believed that the WD-40 repeats mediate protein-protein interactions and are likely to play a role in the control of cytotypic differentiation. None have known enzymatic activity. Members of the family are involved in signal transduction, RNA processing, gene regulation, vesicular trafficking and cytoskeletal assembly. There are

also several members of the family whose function is unknown..
The two orthologues of C21 belong to this group with no known
function. The fly orthologue of C21, *ebi* (Dong et al, 1999),
encodes an evolutionarily conserved protein with a unique
amino terminus, distantly related to F-box sequences, and
tandemly arranged carboxyl-terminal WD40 repeats. Genetic
evidence indicates that *ebi* is involved in EGF receptor signal
transduction. The human orthologue, TBL1 (Bassi et al, 1999),
has no known function but is associated with X-linked late-
onset sensorineural deafness.

[0105] As noted above LR11 has a 4145 bp 3' UTR. Both C21
and C12 also have 3' UTRs that exceed 2000 bases and the
published sequence of CL100 has a 3' UTR of >700 bp but may be
considerably longer since the single AATAAA sequence in the 3'
UTR may not be an authentic polyadenylation site (Salamov et
al, 1997). The mean 3' UTR length for all human cDNAs
deposited in public databases is 740 bp (Pesole et al, 1998).
Although the probability of selecting four genes at random and
having all of them exceed the mean is fairly large (0.16), if
3' UTR length is normally distributed, the probability of
three of the four having 3' UTRs that exceed the mean by 3-5
fold is extremely small. The results suggest that long 3'
UTRs are in some way characteristic of genes expressed by
developing hematopoietic cells. The role of 3' UTRs in the

regulation of gene expression is less well understood than
that of 5' UTRs. Both cis- and trans-acting regulatory
effects have been described. 3' UTRs can affect gene
expression by influencing the localization, stability and

5   translation of mRNAs. Sequence length as well as structure
seem critical. A series of structural motifs that influence
RNA stability, etc. have been described (Pesole et al, 2000)
but none of these are present in the sequences that are
reported here. The diverse roles of 3' UTRs have been

10   reviewed in Decker et al (1995) and Rajagopalan et al (1997).
The differential expression of these genes by immature
hematopoietic cells, in contrast to more mature cells,
suggests that these long 3' UTRs may be characteristic of
genes that play a regulatory role during development.

15   **EXAMPLE 2**

Isolation of C21

[0106] C21 was identified in a cDNA library prepared by
subtractive hybridization between CD34+/CD38- and CD34+/CD38+
BMC in an attempt to identify genes expressed in the earliest

20   stages of hematopoietic differentiation. To determine if the
human cDNA could actually be translated into a protein, the
laboratory of the present inventor used it in an *in vitro*
translation system and the results are shown in Figure 7. The
product produced from the cloned cDNA encodes a protein which

has the predicted molecular mass of 56-57 kDa. The homologous mouse gene was also identified.

Genomic Structure of Human hC21

[0107] C21 is located on chromosome 3 between 3q26 and 3q*ter*. The gene occupies ~100 Kb and the coding structure is assembled from 14-16 small exons. The translational start site is located in the 3rd exon and is 50kb downstream from the first exon. The mouse sequence (SEQ ID NO:11), which encodes the amino acid sequence of mouse C21 (SEQ ID NO:12) also is assembled from 16 exons but the full intronic sequences are not yet known.

Expression of C21

[0108] To facilitate analyzing the expression of the C21 proteins, the deduced peptide sequences were used to prepare anti-peptide antibodies directed against the non-identical portions of the two isoforms. Two rabbits were immunized with each of the carboxyl terminal peptides of C21α and C21β. The deduced sequences of the C-terminal peptides are shown below.

C21 α   TQTGALVHSYRGTGGIFEV**CWNA(C)AGDKVGASASDGSVCVLDLR**

       (SEQ ID NO:17)

C21 β   TQV**CLHYLNGQVLLNLGRSI**CLYTLPHHLVVIPLVALIELLVLK

       (residues 471 to 514 of SEQ ID NO:4)

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[0109] The rabbits were immunized with the peptides having the sequence shown in bold face above coupled to ovalbumin in Freund's Adjuvant (CFA) and boosted with the same antigens in Incomplete Adjuvant every 2 weeks for a total of 4 injections.

5   Antibody titers were measured by ELISA using both the immunizing peptide and the recombinant C21. Since the peptide used to produce the antibody for C21α is identical to the homologous peptide found in TBL1, the necessary specificity could not be demonstrated. Therefore, a new antibody directed

10 against the region of C21 (amino acids 117-125 of SEQ ID NO:2) that differed most from TBL1 was prepared. The resultant antibody was then absorbed with a peptide, CGVSHQNPSK-amide (SEQ ID NO:13) coupled to acrylamide, representing the equivalent sequence in TBL1, to further reduce the

15 possibility of cross-reactivity. The resultant antibody was then affinity purified using the immunizing peptide. The site recognized by this antibody is 30 amino acids downstream of the putative F-box in the amino terminal half of C21. Figure 8A-8D shows Western blots obtained with these antibodies. The

20 anti-C21 β antibodies consistently detect a single band with the molecular mass (~56 kD) predicted by the sequence of the ORF (Figure 8A). Both the new affinity purified antiserum and the anti-C21α antiserum identify the same 56 kDa peptide, but they produce more complex patterns that vary from tissue to

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tissue. Additional components are found at ~60 kDa and ~75

kDa (Figures 8B-8C). The addition of soluble peptide to the

diluted antiserum inhibited the staining of all three bands by

the affinity-purified antibody (Figure 8B). In bone marrow

5   the predominant form is the 56 kDa peptide. Cell lines (293T

and HL60) express readily detectable quantities of C21. In

general, in these cell lines only a single major band is

detected. The results obtained with 293T cells are

particularly striking since anti-C21α produces a single band

10   at ~60 kDa , while anti-C21β detects only a smaller band at 56

kDa (Figure 8C). The results obtained with the affinity

purified antibody are shown in Figure 8D, where this antibody,

specific for C21, is directed at an epitope shared by both

isoforms and detects both bands. The results in Figures 8C

15   and 8D are particularly informative since they: 1) prove that

more than one form of C21 exists, 2) show that the β-isoform

identified by 5' RACE encodes an expressed protein, 3) show

that a single cell type produces more than one isoform, 4)

demonstrate that the higher molecular weight form detected by

20   the antibody to the C-terminal fragment of the α form is C21

and not TBL1 (since the peptide used for the immunization of

the animals that made the affinity absorbed antibody is not

present in TBL1), and, finally, 5) they confirm the

specificity of the staining shown in Figures 9A-9H.

C21 Expression in Normal Human Tissues

[0110] C21α and C21β are expressed by hematopoietic cells in adult bone marrow and fetal liver (Figures 9A-9D). C21α, the isoform originally detected in the subtracted library, stains fetal liver hematopoietic cells with far greater intensity than does C21β. The reverse is true in adult bone marrow where C21α stains a very small fraction of the cells (<0.5%) while C21β stains ~5% of the cells with high intensity (Figures 9C-9D). These results indicate that the expression of the two isoforms is developmentally regulated and suggest that there is a reciprocal relationship between the expression of the α-isoform that is prominent in fetal tissues and the β-isoform that is more highly expressed in the adult. Pre-immune serum from these rabbits did not stain these hematopoietic cells (Figures 9E-9H) and the staining that was observed was blocked by incubating the diluted antiserum (1:200) with the immunizing peptide (1 ug/ml; data not shown). Both C21α and C21β stain distal convoluted tubules and collecting ducts in adult kidney, but neither stains fetal kidney (data not shown). C21β selectively stains the capillary endothelium of the glomeruli while C21α does not. Antibodies to both isoforms also stain a small number of other normal tissues. These include Paneth cells in the stomach and Leydig cells and primary spermatogonia in the testis. No

staining was observed with parenchymal cells of the heart, lung, brain, liver, small and large intestines and pancreas.

[0111] Expression of C21 at the mRNA level has also been detected. Semi quantitative RT-PCR showed that C21 was expressed at high levels in CD34+/CD38- cells (data not shown), and cell lines derived from human hematopoietic tumors (Figure 4B) (Neer et al, 1994). Northern analyses produced a more complicated result. A probe from the 3' untranslated region detected a 7.3 kb transcript in many tissues. The expression of this 7.3 kb message does not correlate with the pattern of protein expression shown in Figures 8A-8D, suggesting that this message is either not translated into protein (possible unspliced) or that its protein product is extremely unstable and therefore present at very low steady state levels. Hybridization with a probe restricted to the open reading frame (ORF) revealed a different pattern. It is shown on the lower half of Figure 10. In addition to the 7.3 kb band there is an abundant 4.7 kb message that is expressed predominantly in hematopoietic tissues and appears to correlate with the protein patterns shown above. Mouse tissues show a similar pattern.

Effect of Over-Expression of hC21α in Mouse Fibroblasts

[0112] Two clones of C21-3T3 (21.9 and 21.5) were selected for study. Both ribonuclease protection assays (Figure 11A)

and Western blotting (Figure 11B) showed that the transfected

cells were actually expressing C21. The probe used in the RPA

to demonstrate the expression of the human mRNA was selected

so that the human product could be distinguished from the

5   endogenous mouse message. The protected fragment is 180 bp.

The anti-C21α peptide antiserum used in the Western blot is

directed against a region of the molecule that is identical in

man and mouse. Since 3T3 cells express mouse C21 and this has

the same MW as the human protein, only quantitative

10   differences could be demonstrated.

[0113] The transfected 3T3 cells showed a dramatic

alteration in their growth properties. They did not show

altered cell-cycle parameters. The proportion of cells in S

phases in exponentially growing cells and in cells approaching

15   their saturation density is the same. What was striking was

differences in the plating efficiency of the transfected and

control cells. The control 3T3 cells, grown in 5% bovine

serum, plate with an efficiency of ~20%. In the same medium,

transfected cells plate at 3-5 fold higher efficiency. There

20   are no differences in either the rate of attachment or the

degree of spreading of the transfected cells compared to the

controls. If, however the cells are maintained in suspension

before plating, the control cells die more rapidly than the

C21 transfected cells, suggesting that C21α might increase the

plating efficiency of the transfected cells by protecting them

from apoptosis. Direct testing confirmed this hypothesis.

The rate of apoptosis in cells grown under conditions of serum

starvation was measured. An example of the results is shown

5   in Figures 12A and 12B. PI staining of DNA was used as a

marker of cell death. Annexin V, which stains phosphatidyl

serine after it is translocated to the exterior surface of the

cell membrane, was used to measure apoptotic cells. Cells in

the lower right quadrant have initiated the apoptotic cascade

10   and are stained with Annexin V but have not lost membrane

integrity and so do not stain with PI. The effect of this

resistance is to prolong the survival of cells in a hostile

environment. The proportion of live cells after 24 hours of

serum starvation is more than 50% greater in the cells over-

15   expressing C21 than in the control cells. The reduction in

apoptotic cells accounts for all of the increased survival.

The protection is not permanent and by 48 hours, the effect on

viability became marginal but the number of apoptotic cells

was still reduced. By 72 hours, the differences were lost.

20   Thus, over-expression of C21α delays, but does not prevent

apoptosis in 3T3. This is consistent with the suggestion that

the mechanism through which C21 acts to delay apoptosis is the

stabilization of an NHR-co-repressor complex.

Influence of Apoptotic Signals on the Intracellular
Localization of C21

[0114] To determine the intracellular localization of C21,
the anti-peptide antibodies described above were used in an
5   indirect immunofluorescence assay. The results are shown in
Figure 13. C21α stains 3T3 more brightly than C21β. The
staining is primarily cytoplasmic (Figure 13, row 2). After
treatment with CPT (row 1), the intensity of the staining
increased. Both cytoplasmic (with perinuclear
10   intensification) and nuclear staining increased. In serum-
starved cells, staining with both antibodies became
predominantly nuclear. The increase in nuclear staining
suggests that under conditions of stress C21 levels increase,
and suggests a physiologic role for C21 in the response to
15   these apoptotic stimuli.

Transgenic Mice

[0115] Because C21 is expressed at high levels in fetal
hematopoietic tissues and in cell lines originating from
hematologic malignancies, it was thought important to examine
20   the effects of over-expression of C21 in hematopoietic cells.
The consequences of over-expression of human C21 in mice have
begun to be examined using transgenic technology. The hC21α
transgene, after transfer to the HS/*vav* vector was excised,
purified, and introduced into the inbred C57BL/6J mouse genome

by pronuclear microinjection. To make HS/*vav* C21, it was

necessary to eliminate the two HinDIII sites from the hC21

cDNA. The variant was made by PCR-based silent mutagenesis in

which caagcttatagagataagcttgca (SEQ ID NO:14) was changed to

5   caggcttatagagataaacttgca (SEQ ID NO:15). The mutant hC21 cDNA

was then digested with BamHI and NotI and ligated into the

pCDNA4 HIS vector. The DNA sequence was determined and

protein expression was confirmed. The cDNA containing hC21

mutant sequence, an upstream translation enhancer and the HIS

10   tag sequence was re-amplified by PCR using proof reading

enzyme using pCDNA 4-21 mutant vector as a template and

ligated into a blunted HS/*vav* vector (Ogilvy et al, 1999).

The orientation of the insert was determined by analyzing the

results of a digestion with Sal1. Plasmids with the correct

15   insert were used to prepare the DNA for blastocyst injection

into the inbred C57BL/6J mouse genome by pronuclear

microinjection.

[0116] Transgenic pups were identified by PCR on tail DNA,

by using primers specific to the SV40 pA sequence. Both of

20   the original pups were females and so additional breeding was

required before homozygous recombinants could be obtained.

Several litters have now been obtained. The homozygotes are

viable but 4-week-old animals have distinctly abnormal white

counts. Granulocytes are present in normal numbers but do not

mature normally. Segmented neutrophils are reduced by almost

90%. These results are summarized in Table 1 below. Only

male mice have been used since the Jackson labs indicate that

there is a sex difference in the proportion of granulocytes

5   (males=28.8±2.8: females=16.6±2.3)I n C57Bl/6 mice . The

results demonstrate that over-expression of C21α has a

profound effect on granulocyte development. The smears of

these mice resemble those reported for mice in which the RAR

gene was knocked out. Myeloid progenitors (Colony-forming

10   Units; CFU) including G, GM and GEMM colonies are present in

these mice but enough animals have not been examined to

determine their frequency.

TABLE 1
Differential Counts of Peripheral Blood WBC in C21 Transgenic
15   **Mice**

		Segmented PMN	Immature Neutrophils	LYMPH	MONO	EOS	BLASTS
C21 TRANSGENIC	mean	10.1	18.3	61.7	4.9	1.2	4.7
	S.D.	4.8	2.6	6.6	1.5	0.8	3.8
WILD TYPE	mean	30.8	6.6	58.0	5.6	0.8	0.0
	S.D.	7.3	2.4	9.2	1.5	0.3	0.0

Interaction of C21 with Transcriptional Regulators

[0117] The interaction of C21 with components of the

20   transcription regulatory complex can be demonstrated by both

GST "pull-down" and co-precipitation.

GST Pull-Down

[0118] Labeled C21 prepared by in vitro translation using 35S methionine can be "pulled-down" by immobilized SMRT. As shown in Figure 14A, full-length C21 is pulled down by GST

5   SMRT but does not bind to a control GST column. Truncated forms of C21 lacking the F-Box (21[δF-box]) or just the N-terminal 40 amino acid (21[δN]) are not pulled down by GST-SMRT (Figure 14B) indicating that both the putative F-box and the N-terminus are critical for the high affinity interaction

10   of C21 with SMRT. A fragment (21[δC]) consisting only of the first 230 amino acids (i.e., lacking all of the WD40 repeats and the carboxyl terminus) was as efficient as the full-length protein in reacting with SMRT. These results not only demonstrate the importance of the N-terminal end of C21 for

15   the interaction with the co-repressors, but highlight the importance of identifying the protein(s) that react with the WD-40-rich carboxyl end of C21.

Co-Precipitation of C21 with HDAC

[0119] Figure 15 shows that C21 associates with HDAC3. In

20   this experiment the cells were transfected with HDAC and C21. The HDAC3 in both lysates was adsorbed to Ni-agarose columns. When present C21 binds to the bound HDAC. The flag-tagged C21 does not bind to Ni-agarose columns that have not bound HDAC. While these data do not show a direct interaction between C21

and HDAC, they demonstrate a complex containing HDAC, will
bind C21 when both proteins are expressed in the same cells.

Effect of Co-Transfection of C21 on SMRT Expression

[0120] SMRT expression is greatly increased in cells
expressing both SMRT and C21. Truncated forms of C21 (21[δC]
and 21[δ N+F-box]) are more effective than the wild type
molecule (Figure 16). These results indicate that the
mechanism by which C21 increases SMRT expression is complex.
They provide evidence that C21 is serving some adaptor
function, linking two molecules, whose interaction regulates
SMRT expression. Elimination of either end ("docking site")
creates molecules that interfere with this regulation and lead
to large increases in C21 expression. The effect is seen with
the fragment that binds SMRT (21[δC]) and one that has no
affinity for SMRT (21[δ N+F-box]). However, since co-
transfection with the wild type C21 also leads to a
significant increase in SMRT expression, it appears that the
region between amino acids 80 and 175 may also play an
autonomous role increasing SMRT expression. If this is
correct, then it is likely that primary role of C21 is the
regulation of proteasomal targeting and that the amino
terminal end docks the molecule to the co-repressor; the
central portion interacts with the ubiquitination pathway and
the carboxy-terminal provides tissue specific activation.

[0121] The expression of the C21 mutants varies considerably. The most protection was produced by co-transfection with C21δNf, which was also the most highly expressed C21 protein. However the next most effective protein was C21δC which was the least expressed of the three C21 proteins. Co-transfection with siah-1, a factor known to contribute to the targeting of N-CoR for proteolytic destruction did not affect SMRT expression. COS 7 cells were co-transfected with pCDNA His-SMRT (1-300) and the His-tagged C21 constructs described above. Control cells were transfected with pCDNA His-SMRT (1-300) and an empty vector.

Effect of Co-Transfection of C21 on SMRT Stability

[0122] Co-transfection of C21 with SMRT retards the degradation of SMRT. The results of a pulse-chase experiment showing this are in Figure 17. In control 293T cells the ^{35}S SMRT is undetectable after 3 hours and barely detectable after 1 hour, while in cells transfected with C21, labeled SMRT was easily detected throughout the experiment. The difference in the intensity of the labeling at 0 time reflects the instability of the labeled SMRT in the control cells. Much of the material labeled during the pulse is degraded during the labeling period. The band marked *** is an unknown protein that co-purified with SMRT. The label in this protein is

stable and the intensity of the band serves as an internal control for loading etc.

Effect of Proteasome Inhibitors on SMRT Expression

[0123] To demonstrate the levels of SMRT found in COS 7 cells are controlled by ubiquitination the transfected cells were treated with MG132 in DMSO or with DMSO as a control and the level of SMRT was measured by Western blotting as described above. MG 132 (N-CBZ-Leu-Leu-Leu-al) is a potent, membrane-permeable proteasome inhibitor (Lee et al, 1998). To control for differences in cell survival etc., the COS cells were co-transfected with a vector that expressed His-GFP along with the His-SMRT vector. GFP is not degraded via the ubiquitin-proteasome pathway. As shown in Figure 18, MG132 treated COS7 cells express levels of SMRT that are ~25 times those found in the DMS) control. Only a small difference in GFP expression was observed.

[0124] In summary, the present inventor has identified C21 as a novel transcriptional regulator. The gene encoding this protein has been cloned and identified as a member of a small family of proteins that include at least two isoforms encoded by the same gene and another X-linked protein called TBL1. The family members interact with the co-repressors that regulate the activity of hormone-dependent, nuclear transcription factors. The evidence suggests that these

proteins act by altering the stability of the co-repressor

complex and that they may also contribute, or recruit other

proteins that provide, an autonomous repressor function. The

functional effects of altering C21 expression are likely to

5   alter many aspect of cellular metabolism. When over-expressed

in hematopoietic cells, C21α alters the pathway of myeloid

differentiation, inhibiting the formation of mature

granulocytes. When over-expressed in fibroblasts, C21

inhibits the apoptotic response.

10   [0125] Having now fully described this invention, it will

be appreciated by those skilled in the art that the same can

be performed within a wide range of equivalent parameters,

concentrations, and conditions without departing from the

spirit and scope of the invention and without undue

15   experimentation.

[0126] While this invention has been described in

connection with specific embodiments thereof, it will be

understood that it is capable of further modifications. This

application is intended to cover any variations, uses, or

20   adaptations of the inventions following, in general, the

principles of the invention and including such departures from

the present disclosure as come within known or customary

practice within the art to which the invention pertains and as

may be applied to the essential features hereinbefore set

forth as follows in the scope of the appended claims.

[0127] All references cited herein, including journal

articles or abstracts, published or corresponding U.S. or

5   foreign patent applications, issued U.S. or foreign patents,

or any other references, are entirely incorporated by

reference herein, including all data, tables, figures, and

text presented in the cited references. Additionally, the

entire contents of the references cited within the references

10   cited herein are also entirely incorporated by references.

[0128] Reference to known method steps, conventional

methods steps, known methods or conventional methods is not in

any way an admission that any aspect, description or

embodiment of the present invention is disclosed, taught or

15   suggested in the relevant art.

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